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Signal Transduction:

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J. Biol. Chem. 2011, 286:19777-19788.
doi: 10.1074/jbc.M111.237081 originally published online March 28, 2011

THE JOURNAL OF BIOLOGICAL CHEMISTRY VOL. 286, NO. 22, pp. 19777–19788, June 3, 2011

Mislocalization of the E3 Ligase, β-Transducin Repeat-containing Protein 1 (β-TrCP1), in Glioblastoma Uncouples Negative Feedback between the Pleckstrin Homology Domain Leucine-rich Repeat Protein Phosphatase 1 (PHLPP1) and Akt*[S]

Received for publication, March 4, 2011, and in revised form, March 25, 2011 Published, JBC Papers in Press, March 28, 2011, DOI 10.1074/jbc.M111.237081

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The PH domain leucine-rich repeat protein phosphatase, PHLPP, plays a central role in controlling the amplitude of growth factor signaling by directly dephosphorylating and thereby inactivating Akt. The cellular levels of PHLPP1 have recently been shown to be enhanced by its substrate, activated Akt, via modulation of a phosphodegron recognized by the E3 ligase β-TrCP1, thus providing a negative feedback loop to tightly control cellular Akt output. Here we show that this feedback loop is lost in aggressive glioblastoma but not less aggressive astrocytoma. Overexpression and pharmacological studies reveal that loss of the feedback loop does not result from a defect in PHLPP1 protein or in the upstream kinases that control its phosphodegron. Rather, the defect arises from altered localization of β-TrCP1; in astrocytoma cell lines and in normal brain tissue the E3 ligase is predominantly cytoplasmic, whereas in glioblastoma cell lines and patient-derived tumor neurospheres, the E3 ligase is confined to the nucleus and thus spatially separated from PHLPP1, which is cytoplasmic. Restoring the localization of β-TrCP1 to the cytosol of glioblastoma cells rescues the ability of Akt to regulate PHLPP1 stability. Additionally, we show that the degradation of another β-TrCP1 substrate, β-catenin, is impaired and accumulates in the cytosol of glioblastoma cell lines. Our findings reveal that the cellular localization of β-TrCP1 is altered in glioblastoma, resulting in dysregulation of PHLPP1 and other substrates such as β-catenin.

Glioblastomas (World Health Organization grade IV) account for ~70% of all tumors of the central nervous system (CNS), making them the most common type of malignant brain tumor (1). Primary glioblastoma, commonly referred to as glioblastoma multiforme (GBM),[3] accounts for the vast majority of glioblastoma cases and presents in an acute *de novo* manner with no prior evidence of lower grade pathology. Secondary glioblastomas are less common and are derived from the progression of lower grade astrocytomas (World Health Organization I-III) (2). Loss or dysregulation of a tumor suppressor can result in the activation of signaling pathways that drive cell growth, proliferation, and survival and aid tumor initiation and development (3).

One signal transduction pathway that is important to the initiation and progression of many cancer types, including those of the CNS, is the phosphatidylinositol 3-kinase (PI3K)/Akt pathway. In the presence of proliferative signals, Akt is activated by phosphorylation at two crucial sites. The first site, known as the activation loop (Thr-308 on Akt1), is phosphorylated by PDK-1 (4). The second site, termed the hydrophobic motif (Ser-473 on Akt1), is phosphorylated through a mechanism regulated by the TORC2 protein complex (5, 6). Once activated, Akt phosphorylates defined substrates in the cytosol and nucleus, ultimately inducing proliferation and anti-apoptotic signaling pathways (7). Signaling by Akt is terminated by two primary mechanisms; that is, removal of the activating lipid second messenger by the phosphatase PTEN (phosphatase and tensin homolog on chromosome ten) (8) and direct dephosphorylation of the kinase by phosphatases, including PHLPP (9, 10).

A second signaling pathway that is often amplified in cancer is the Wnt/β-catenin signaling pathway, which primarily functions to regulate cell proliferation and apoptosis. Under basal conditions, levels of free, cytosolic β-catenin are suppressed by proteasomal degradation. This process is regulated by a protein complex composed of axin, adenomatous polyposis coli, casein kinase 1 (CK1), and glycogen synthase kinase-3 (GSK-3) (11). Accumulation and nuclear translocation of cytosolic β-catenin

* This work was supported, in whole or in part, by National Institutes of Health Grant GM067946 (to A. C. N.). This work was also supported by Department of Defense Breast Cancer Research Program Award BC093021 (to N. W.).

[S] The on-line version of this article (available at http://www.jbc.org) contains supplemental Tables 1 and 2 and Figs. 1 and 2.

[1] Supported in part by the University of California at San Diego Graduate Training Program in Cellular and Molecular Pharmacology through NIGMS, National Institutes of Health Institutional Training Grant T32 GM007752.

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[3] The abbreviations used are: GBM, glioblastoma multiforme; PHLPP, pleckstrin homology domain leucine-rich repeat protein phosphatase; CK1, casein kinase 1; GSK-3, glycogen synthase kinase-3; β-TrCP, β-transducin repeat containing protein; RFP, red fluorescent protein; CFP, cyan fluorescent protein.

activates various oncogenic substrates including c-Myc, cyclin D1, and members of the AP-1 family (12, 13).

Previous studies have shown that both PI3K/Akt and β-catenin signaling can be up-regulated in tumorigenesis through a number of mechanisms. In the case of Akt, these include gene amplification or gain of function mutations in upstream receptor tyrosine kinase and hormone receptors (the most common mechanism in CNS tumors), activating mutations in PI3K, or loss of function mutations in the regulatory phosphatase PTEN (14–17). In the case of β-catenin, activation can result from amplification of upstream components of the Wnt pathway such as Dishevelled as well as mutations to β-catenin itself and regulatory proteins such as adenomatous polyposis coli and axin (18–20). However, these mechanisms alone do not account for all instances where these signaling pathways are constitutively active in tumors, suggesting that alterations in additional proteins is responsible for activation.

The PHLPP phosphatases are members of a novel family of Ser/Thr phosphatases composed of three isozymes: the alternatively spliced PHLPP1α and PHLPP1β and a separate gene product, PHLPP2 (21). Our laboratory has previously established that PHLPP selectively dephosphorylates the hydrophobic motif of Akt and protein kinase C (PKC) isozymes (9, 22). In the case of Akt, dephosphorylation at this site reduces its intrinsic catalytic activity, leading to increased apoptosis and decreased proliferation (9, 10). In the case of PKC, dephosphorylation destabilizes PKC and shunts it to degradation pathways (22). There is mounting evidence that PHLPP serves as a tumor suppressor protein in cancer. First, overexpression of PHLPP in normal or cancer cells decreases proliferation and induces apoptosis in concert with inactivation of Akt signaling. Furthermore, overexpression of PHLPP1α in a glioblastoma cell line was shown to greatly reduce tumor growth in a nude mouse model (9). Second, PHLPP1 and PHLPP2 are frequently absent or reduced in cancer. Notably, PHLPP1 mRNA has been shown to be reduced by an order of magnitude in chronic lymphocytic leukemia (CLL) (23) and, in fact, was absent in >50% of CLL tumors in one study (24); PHLPP1 and PHLPP2 mRNA levels were found to be 5- and 4-fold lower, respectively, in esophageal adenocarcinomas (25), and several studies have identified significant reduction in PHLPP1 and PHLPP2 mRNA in colon cancer (26–28). PHLPP1 mRNA has also been shown to be 2-fold lower in glioblastoma (29), melanoma (30), and breast carcinomas (31, 32). Additionally, PHLPP1 protein levels are reduced in cancers of the liver, pancreas, and stomach (33). Furthermore, PHLPP1 and PHLPP2 protein expression is significantly decreased or lost in 78 and 86%, respectively, of colon tumors (34). Thus, reductions in PHLPP accompany a broad spectrum of cancers, consistent with loss of PHLPP conferring a survival advantage to cancer cells. Indeed, PHLPP is deleted as often as PTEN (~40%) in metastatic prostate cancers (35). Aberrations in PHLPP may be particularly prevalent in glioblastoma, where several mutations have been found in human tumors and where higher levels of PHLPP correlate with higher survival rates (36, 37). These findings suggest that maintenance of PHLPP levels is important for inhibition of tumor growth, and mechanisms that control the amount of PHLPP in the cell are often lost in tumorigenesis.

Ubiquitin-mediated degradation is important for the proper regulation of many cellular processes including transcription and cell cycle progression. E3 ubiquitin protein ligases confer the specificity of this system by binding and targeting certain substrates for ubiquitination. The levels of E3 ligases can dramatically alter signal transduction pathways involved in tumorigenesis by influencing the levels of their substrate (38). One such E3 ligase family, β-transducin repeat containing proteins (β-TrCPs), are F-box proteins that recognize phosphorylated serine residues of target substrates that are processed by the ubiquitin/proteasome pathway (39, 40). β-TrCP1 has been demonstrated to mediate the phosphorylation-dependent degradation of numerous proteins involved in tumorigenesis, the most well studied being β-catenin (41, 42) and IκB (43). Recently, β-TrCP1 was identified as the E3 ligase responsible for ubiquitin-mediated degradation of PHLPP1 in a phosphorylation-dependent manner (44). Similar to β-catenin, PHLPP1 is directly phosphorylated by CK1 and GSK-3β on multiple serine residues to create a phosphodegron motif. β-TrCP1 then binds PHLPP1 through recognition of this destruction motif, and PHLPP1 is subsequently polyubiquitinated and then degraded by the proteasome (44). Phosphorylation of GSK-3β by Akt inhibits its activity and thus directly influences the stability of PHLPP1; this results in a feedback loop through which active Akt stabilizes its negative regulator, PHLPP1, ultimately serving to dampen Akt signaling.

Here we identify a subset of tumors in which the negative feedback loop by which the level of active Akt determines the expression of its negative regulator PHLPP1 is lost. Specifically, we have found that this feedback loop is broken in high grade glioblastomas because of the confinement of β-TrCP1 to the nuclear compartment, where it can no longer target its cytosolic substrates for degradation, providing a novel mechanism for the dysregulation of PHLPP1 and β-catenin levels.

EXPERIMENTAL PROCEDURES

Plasmids—HA-PHLPP1α has been previously described (9). The additional 1536 base pairs of PHLPP1β were amplified by OneStep RT-PCR (Qiagen) from RNA isolated from human brain and subcloned into the HA-PHLPP1α vector to generate the full-length PHLPP1β gene product (NM_194449) (21). RFP-PHLPP1β was constructed by PCR amplification and subsequent cloning into a pcDNA3 vector with monomeric RFP as an N-terminal tag. Myc-epitope tagged β-TrCP1 was purchased from Addgene and has been described previously (45). β-TrCP1 was subcloned in-frame C-terminal to CFP in pcDNA3 vector using Gateway cloning techniques (Invitrogen).

Materials and Antibodies—Cycloheximide, LY294002, GSK3 inhibitor IX (GSK3i IX), and MG-132 were purchased from Calbiochem and dissolved in dimethyl sulfoxide (DMSO). Antibodies to PHLPP1 and PHLPP2 were purchased from Bethyl Laboratory. The following antibodies were purchased from Cell Signaling: phospho-antibodies for Thr-308 (P308) and Ser-473 (P473) of Akt, phospho-GSK-3α/β (Ser-21/9), phospho-glycogen synthase (Ser-641), phospho-β-catenin (Ser-45), and total Akt Antibody. An anti-HA monoclonal antibody was purchased from Covance. A DsRed (anti-RFP) anti-

body was purchased from Clontech. A β-TrCP1-specific antibody was purchased from Invitrogen. Monoclonal antibodies to actin and Myc were purchased from Sigma. Antibodies to annexin 1, lamin A, and β-catenin were purchased from Santa Cruz Biotechnology. The antibody to voltage-dependent anion channel protein (VDAC) was obtained from Affinity BioReagents. Ultra-link protein A/G beads were obtained from Thermo Scientific. All other materials and chemicals were reagent grade. Non-viable cell pellets from the NCI60 panel of tumor cell lines were provided by the Developmental Therapeutics Program (NCI/National Institutes of Health).

Cell Transfection and Immunoblotting—SNB-75, SF-268, SF-295, SF-539, SNB-19, U251, and H157 cell lines were maintained in RPMI (Cellgro) containing 10% FBS (Hyclone) and 1% penicillin/streptomycin at 37 °C in 5% CO_2. Transient transfection of all cell lines was carried out using jetPRIME transfection reagent (Polyplus Transfection) following the manufacturer's protocol. For immunoblotting, cultured cells and non-viable cell pellets were lysed in buffer A (50 mM Na_2HPO_4, 1 mM sodium pyrophosphate, 20 mM NaF, 2 mM EDTA, 2 mM EGTA, 1% Triton X-100, 1 mM DTT, 200 μM benzamidine, 40 μg ml^{-1} leupeptin, and 1 mM PMSF, pH 7.4) and sonicated for 5s, and protein yield was determined using the Coomassie BCA protein assay (Pierce). Lysates containing equal protein were analyzed by SDS-PAGE, and individual blots were probed using the indicated antibody. Densitometric analysis was performed with AlphaView analysis software (Version 1.3.0.6) by Alpha Innotech Corp.

Cellular Ubiquitination Assays—H157 cells were transfected with DNA encoding RFP-PHLPP1β (1 μg) and HA-ubiquitin (0.5 μg). Cells were pretreated for 30 min with MG-132 (10 μM) before the addition of LY294002 (20 μM) or DMSO for the indicated times before harvest with immunoprecipitation buffer (50 mM Tris-HCl, pH 7.4, 100 mM NaCl, 5 mM EDTA, 1% Triton X-100, 10 mM sodium pyrophosphate, 1 mM phenylmethylsulfonyl fluoride, 1 mM sodium vanadate, and 10 mM *N*-ethylmaleimide to preserve the ubiquitinated species). Ten percent of the total detergent-solubilized cell lysate was quenched in SDS sample buffer for further analysis, and the remaining lysate was cleared by centrifugation at 13,000 × *g* for 5 min. For immunoprecipitation, the supernatants were incubated with DsRed (anti-RFP) antibody overnight at 4 °C and then with Ultra-link protein A/G beads for and additional 2 h. The immunocomplexes were washed three times with immunoprecipitation buffer, and proteins were separated by SDS-PAGE and analyzed by immunoblotting.

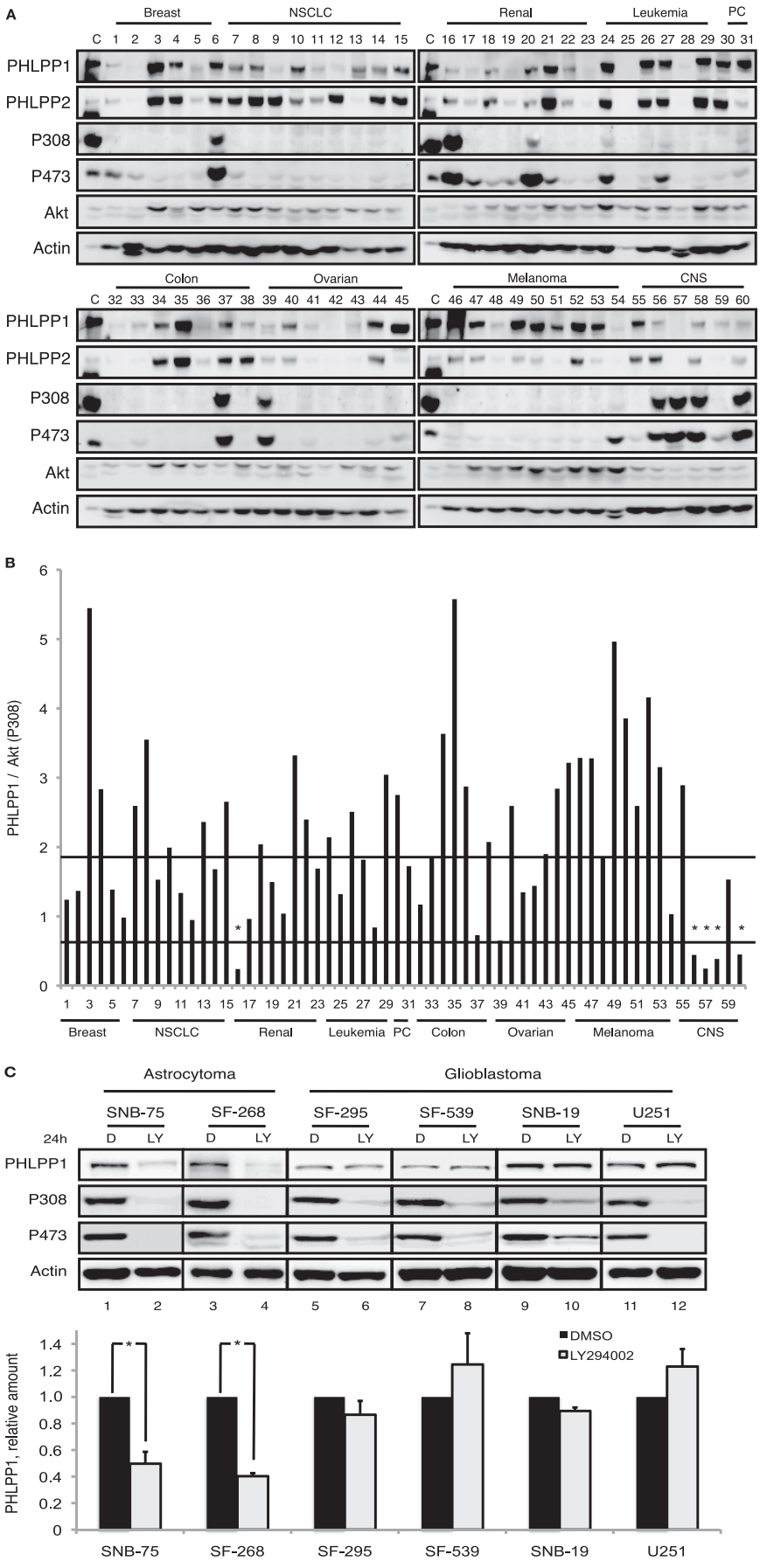
Cellular Fractionation—Tissue samples from healthy human brain (case numbers 1505 and 1530) were homogenized at low speed and fractionated using the Qproteome Cell Compartment kit (Qiagen) according to the manufacturer's protocol. Astrocytoma, glioblastoma, and patient-derived glioblastoma tumor neurosphere cell lines were fractionated by centrifugation as follows. Approximately 1×10^6 cells were lysed in 200 μl of hypotonic lysis buffer B (10 mM Tris-HCl, pH 7.5, 50 mM NaCl, 1 mM sodium pyrophosphate, 20 mM NaF, 200 μM benzamidine, 40 μg/ml leupeptin, 1 mM PMSF, and 2 mM sodium vanadate). Lysates were passed through a 25-gauge needle 12 times, and a portion of the lysate was retained; the remainder was centrifuged at 800 × *g* for 5 min at 4 °C to pellet the nuclear material. The nuclear pellet was then resuspended in 100 μl of buffer C (buffer B + 1% Triton X-100), mixed by vortex, and placed on ice for 10 min before centrifugation at 16,000 × *g* for 15 min. The resulting supernatant was collected as the nuclear fraction. The supernatant taken after isolation of the nuclear pellet was subjected to centrifugation for 20 min at 110,000 × *g*. The resulting supernatant was collected as the cytosolic fraction. The remaining pellet was resuspended in 100 μl of buffer C, mixed by vortex, and placed on ice for 10 min before centrifugation at 110,000 × *g* for 20 min. The resulting supernatant was collected as the membrane fraction. The remaining pellet was resuspended in 100 μl of buffer C and collected as the detergent-insoluble pellet. An equal volume of each fraction was run on an SDS-PAGE gel and analyzed by immunoblotting.

Generation of Neurospheres—Eligible patients were those undergoing craniotomy for therapeutic management of a brain tumor. Written informed consent was obtained from all patients. The study was approved by the Lothian (Scotland, UK) regional ethics committee (LREC 2004/4/16). Tumor biopsies were processed within 1 h. Cell lines were expanded from gliomas as described previously (46, 47). Briefly, tissue was homogenized and trypsinized. The single cell suspension was pelleted then resuspended in expansion media (Advanced DMEM:F-12 (1:1), 1% B27 (10×), 0.5% N_2 (100×), 1% 100 mM Glutamax, 1% penicillin-streptomycin, 1% Fungizone, 10 ng/ml EGF (R&D Systems, Abingdon, UK), 10 ng/ml basic fibroblast growth factor (R&D Systems), 5 μg/ml heparin (Sigma)), in a Matrigel-coated (BD Biosciences) flask. All reagents and products are from Invitrogen unless otherwise stated. Experiments were performed on low passage number cells (less than passage 10). Patient-derived neurosphere cell lines (named O, U, and P) were designated GBM1, GBM2, and GBM3, respectively, and two additional neurosphere cell lines (TS600 and TS576) have been described previously (48).

RESULTS

Akt-mediated Enhancement of PHLPP1 Is Preferentially Lost in Glioblastoma—The frequent elevation of Akt activity in cancer, often via unexplained mechanisms, led us to explore whether the feedback loop between Akt and PHLPP1 is lost in certain tumors. To this end we compared the activity of Akt, assessed via the phosphorylation state of Thr-308, with the levels of PHLPP1 in the NCI60 panel of tumor cell lines (Fig. 1*a*). Based on the enhancement of PHLPP1 levels by active Akt (thus creating a feedback loop to suppress Akt activity) (44), PHLPP1 levels were predicted to track with Akt activity in cell lines containing an intact feedback loop. However, in cells where this feedback loop is broken, an inverse correlation between PHLPP1 levels and Akt activity would be expected. The Western blots in Fig. 1*a* present the levels of PHLPP1[4] and PHLPP2, the phosphorylation state of Akt on Thr-308 and Ser-473, and the relative amount of Akt in lysates from each of the NCI60 cell lines. The first lane of each gel was loaded with an equal amount

[4] This is the first reported cloning of the human PHLPP1β isoform; throughout this study, PHLPP1 refers to PHLPP1β because it is the predominant isoform expressed in all cell lines examined.



of lysate from COS7 cells overexpressing PHLPP1 to serve as an internal control for differences in exposure between immunoblots. To compare the level of PHLPP1 to Akt activity in each cell line, we plotted the ratio of PHLPP1 to Thr-308 phosphorylation (Fig. 1*b*). Note that the phosphorylation of Akt on Thr-308 was chosen to represent Akt activity because phospho-Ser-473 is a direct substrate of PHLPP and could potentially be regulated by the feedback loop described here or be elevated because of a cellular defect in PHLPP itself. However, in most cases, the relative amount of phospho-Thr-308 mirrored that of phospho-Ser-473. We identified 5 tumor cell lines (indicated by *asterisks* in Fig. 1*b*) in which the ratio of PHLPP1:Akt (Thr-308) was at least 1 S.D. below the median value (2 ± 1) of all 60 cell lines. These tumor cells have low levels of PHLPP1 despite very high basal Akt activity, consistent with potential loss of the Akt-mediated feedback loop on PHLPP1. Four of the five identified cell lines are defined as glioblastomas (SF-295 (number 56), SF-539 (number 57), SNB-19 (number 58), and U251 (number 60); designated cell line numbers are listed in supplemental Table 1), and the other is an adenocarcinoma derived from a renal tumor (786–0 (number 16)). Interestingly, the four CNS cell lines with a low PHLPP1:Akt (Thr-308) ratio (numbers 56, 57, 58, and 60) are all classified as glioblastomas, whereas the two CNS lines predicted to have an intact feedback loop (numbers 55 and 59) are less aggressive astrocytomas.

If the feedback loop is lost in the candidate glioblastoma cell lines with elevated Akt activity and low PHLPP1 expression (Fig. 1*b*), we reasoned that Akt activity would no longer affect the stability of PHLPP1 in these cell lines. However, in the less aggressive astrocytoma cell lines in which the ratio of PHLPP1 to Akt (Thr-308) would indicate that the feedback loop is intact, PHLPP1 levels would be expected to retain sensitivity to pharmacological manipulation of Akt activity. To test this hypothesis, we treated both astrocytoma cell lines and the four candidate glioblastoma cell lines with DMSO or a PI3K inhibitor (LY294002) for 24 h and then analyzed PHLPP1 levels. Fig. 1*c* reveals that LY294002 effectively decreased the steady-state levels of PHLPP1 by >50% in both astrocytoma cell lines (*e.g.* compare *lanes 1* and *2*) but had no significant effect on the steady-state levels of PHLPP1 in the glioblastoma cell lines tested (*e.g.* compare *lanes 5* and *6*). Thus, we have identified a subset of aggressive CNS tumors that have lost Akt-mediated control on the stability of PHLPP1, a key regulatory mechanism for homeostasis of the Akt signaling output.

Loss of the Feedback Loop in Glioblastoma Is Independent of PHLPP1, CK1, and GSK-3β—As previously reported (44), the effects of Akt activity on PHLPP1 stability are mediated through GSK-3β-dependent phosphorylation of PHLPP1, resulting in its ubiquitination and proteasome-mediated degradation. Consistent with this mechanism, inhibition of PI3K by LY294002 caused a robust increase in the ubiquitination of PHLPP1 (supplemental Fig. 1), whereas inhibition of GSK-3 or the proteasome prevented the LY294002-induced reduction in PHLPP1 steady-state levels (supplemental Fig. 2).

To determine whether a defect in PHLPP1 itself is responsible for the loss of Akt-mediated regulation of its stability, we monitored the stability of an exogenously expressed construct of PHLPP1 in response to PI3K inhibition. HA-tagged PHLPP1 was transfected into an astrocytoma cell line (SF-268) and several of the candidate glioblastoma cell lines (SF-295, SNB-19, U251) for 24 h before treatment with DMSO or LY294002 for 24 h; anti-HA antibody was used to monitor levels of exogenous PHLPP1 (Fig. 2*a*). The activity of Akt was effectively blocked by treatment with LY294002 in all cell lines, as indicated by the reduced phosphorylation of Akt (Ser-473). Similar to endogenous PHLPP1 in the astrocytoma cell line containing an intact feedback loop, exogenous PHLPP1 levels in these cells were also significantly reduced after Akt inhibition (compare *lanes 1* and *2*). However, the level of exogenous PHLPP1 was refractory to Akt inhibition in all three of the glioblastoma lines, which harbor a broken feedback loop (*e.g.* compare *lanes 3* and *4*). The inability to rescue the defect by overexpression of exogenous PHLPP1 reveals the loss of Akt-mediated regulation of PHLPP1 in glioblastoma is not caused by a defect in PHLPP1 itself.

We next sequentially investigated the functionality of each player involved in the described phosphorylation-dependent degradation pathway from Akt to PHLPP1 to identify at which step the loop is broken in the indicated glioblastoma cell lines (Fig. 2*b*). First, we tested whether Akt activity was being effectively blocked by inhibition of PI3K in glioblastomas and astrocytomas. In addition to loss of phosphorylation at Ser-473, phosphorylation of an Akt substrate, GSK-3α/β (Ser-21/9), was reduced in response to Akt inhibition by similar amounts in both astrocytoma (compare *lanes 1* and *2*) and glioblastoma cell lines (*e.g.* compare *lanes 3* and *4*). These data demonstrate that inhibition of PI3K is able to decrease Akt activity in both astrocytoma and glioblastoma cells (Fig. 2*b*). Second, we tested whether GSK-3β is able to properly phosphorylate its substrates in cells with a broken feedback loop. To this end, we examined the phosphorylation state of glycogen synthase, an established target of GSK-3β, after 24 h of PI3K inhibition. After relief of the Akt-mediated inhibition of GSK-3β, phosphorylation of glycogen synthase (Ser-641) was increased in both glioblastoma and astrocytoma cell lines, indicating that GSK-3β is functional toward its substrates (Fig. 2*b*). Third, we investigated whether CK1 is able to phosphorylate its substrates in glioblastoma. The stability of a well documented CK1 substrate, β-catenin, is regulated in a manner analogous to that

FIGURE 1. **Akt-mediated feedback loop enhancing PHLPP1 levels is preferentially lost in CNS tumors.** *A*, lysates of non-viable cell pellets from the NCI60 panel of tumor cell lines (*NSCLC*, non-small cell lung cancer; *PC*, prostate cancer; *CNS*, central nervous system) were analyzed by Western blotting for PHLPP1, PHLPP2, Akt phosphorylation on Thr-308 (*P308*) or Ser-473 (*P473*), Akt, and actin. The *first lane* in each gel (*C*) contains equal lysate from COS7 cells overexpressing PHLPP1 and serves to control for differences in exposure among different blots. *B*, the ratio of PHLPP1 to active Akt (Thr-308) was obtained by densitometric analysis of the blots in *a* and plotted across the panel of 60 cell lines. *Horizontal lines* indicate the value of the median (*top line*) and one S.D. below the median value (*bottom line*). Cell lines with a ratio less than 1 S.D. below the median line are indicated by an *asterisk*. *C*, the indicated CNS cell lines were treated for 24 h with DMSO (*D*) or LY294002 (*LY*) (20 μM) and PHLPP1, Akt phosphorylation on Thr-308 (*P308*) or Ser-473 (*P473*), and actin were detected by Western blot analysis. The relative amount of PHLPP1, normalized to actin, is shown in the graph; data represent the mean ± S.E. of three independent experiments.

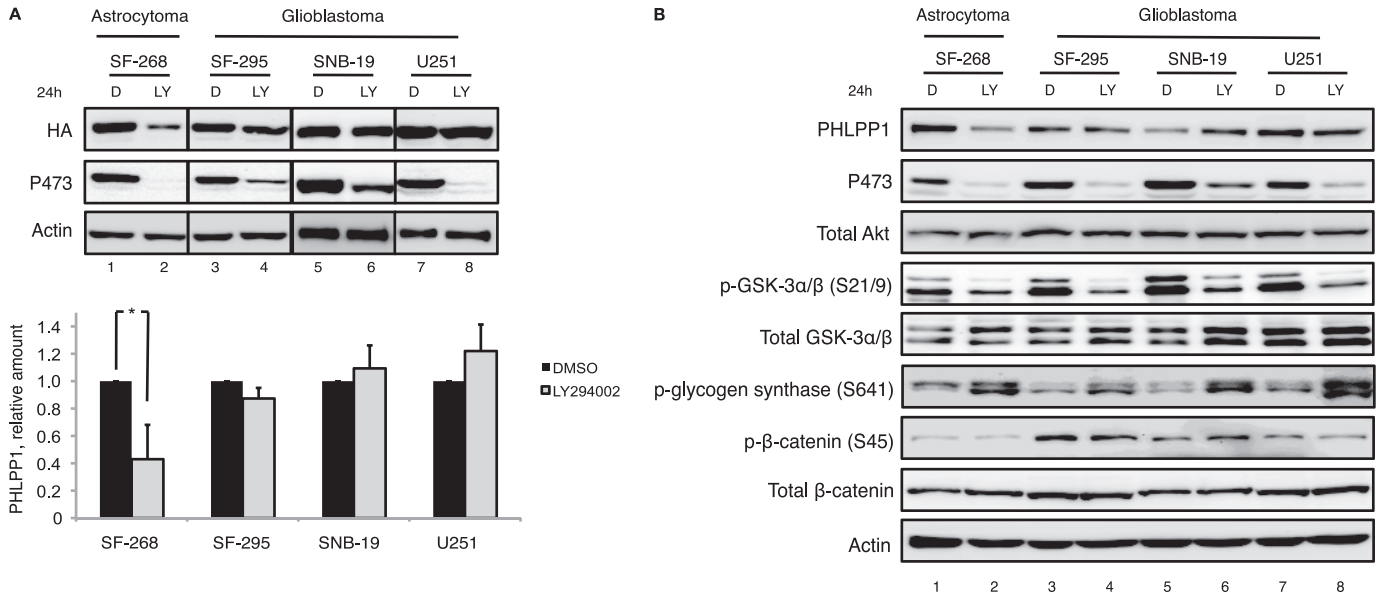


FIGURE 2. **Loss of the feedback loop in glioblastoma is independent of PHLPP1, CK1, and GSK-3.** *A*, astrocytoma and glioblastoma cell lines were transfected with HA-PHLPP1 for 24 h before 24 h treatment with DMSO or LY294002 (20 μM). Immunoblotting was used to determine levels of exogenous PHLPP1 (*HA*) as well as the phosphorylation state of Akt (Ser-473) and actin. The relative amount of exogenous HA-PHLPP1, normalized to actin, is shown in the graph; data represent the mean ± S.E. of three independent experiments. *, $p < 0.05$ compared with DMSO. *B*, the indicated cell lines were treated with DMSO or LY294002 (20 μM) for 24 h, and immunoblotting was used to determine PHLPP1 levels, the phosphorylation state of Akt (Ser-473), phospho-GSK-3α/β (Ser-21/9), phospho-glycogen synthase (Ser-641), and phospho-β-catenin (Ser-45). Actin was used as a loading control.

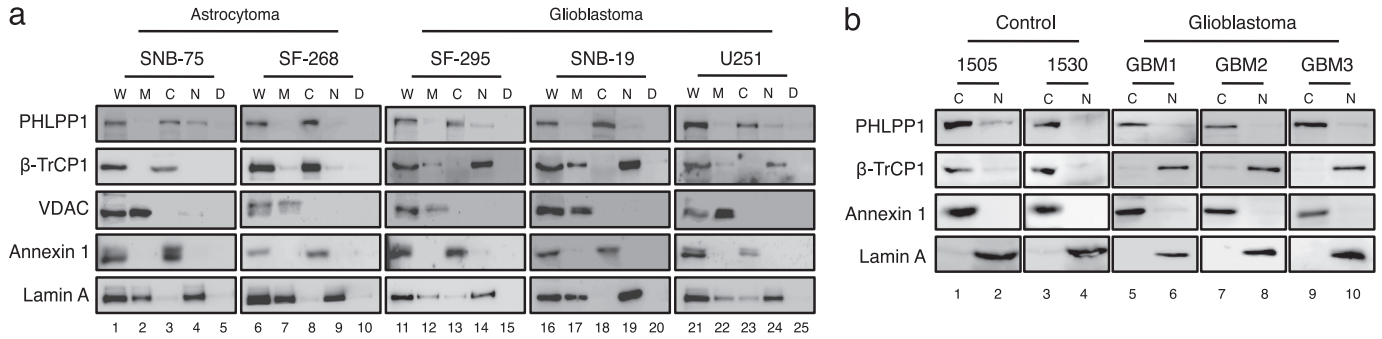


FIGURE 3. **β-TrCP1 is confined to the nucleus in glioblastoma cell lines and patient samples.** *a*, astrocytoma and glioblastoma cell lines were fractionated as described under "Experimental Procedures." Fractions representing equal amounts of cell lysate were analyzed by immunoblotting to determine the cellular location of PHLPP1 and β-TrCP1. *b*, frontal cortex from healthy human brain and patient-derived glioblastoma tumor neurosphere specimens were fractionated as described under "Experimental Procedures." Fractions were analyzed by immunoblotting to determine the cellular location of PHLPP1 and β-TrCP1. Voltage-dependent anion channel protein (membrane) (*VDAC*), annexin 1 (cytosol), and lamin A (nucleus) were used as control markers for the indicated cellular fractions. *W*, whole cell lysate; *M*, membrane; *C*, cytosol; *N*, nuclear; *D*, detergent-insoluble pellet.

of PHLPP1, where a constitutive "priming" phosphorylation by CK1 is necessary for subsequent phosphorylation within the phosphodegron motif. Therefore, to determine whether CK1 is functional in glioblastoma, we probed the CK1 site on β-catenin (Ser-45) with a phospho-specific antibody. We observed a basal level of β-catenin (Ser-45) phosphorylation in all of the cell lines tested, indicating that CK1 is functional in both intact and broken loop cell lines (Fig. 2*b*). Taken together, these data indicate that loss of Akt-mediated regulation of PHLPP1 stability does not result from a defect in PHLPP1 or the kinases responsible for phosphorylating and promoting its degradation.

β-TrCP1 Is Confined to the Nucleus in Glioblastoma Cell Lines and Patient Samples—Having established that the upstream kinases responsible for regulation of PHLPP1 stability are functional, we next asked whether a defect in the E3 ligase and/or proteasomal degradation machinery could be responsible for the broken feedback loop in glioblastoma. One possible mechanism for the loss of Akt-mediated regulation of PHLPP1 in glioblastoma is that the cellular localization of PHLPP1 or its E3 ligase, β-TrCP1, could be altered in these cells. To examine the localization of these proteins, astrocytoma and glioblastoma cell lines were lysed, and the membrane, cytoplasmic, nuclear, and detergent-insoluble fractions were isolated. Equal portions of starting material were then analyzed by immunoblotting to determine the subcellular location of PHLPP1 and β-TrCP1. To verify that we effectively isolated the indicated cellular fractions, antibodies specific to proteins that reside in each fraction were used as a control (Fig. 3*a*). Although the fractionation was clean for the most part, we did observe some contamination between our nuclear and membrane fractions, as revealed by the presence of lamin A, a nuclear protein, in the membrane fraction. However, it is important to note that there was no contamination between the cytosolic and nuclear frac-

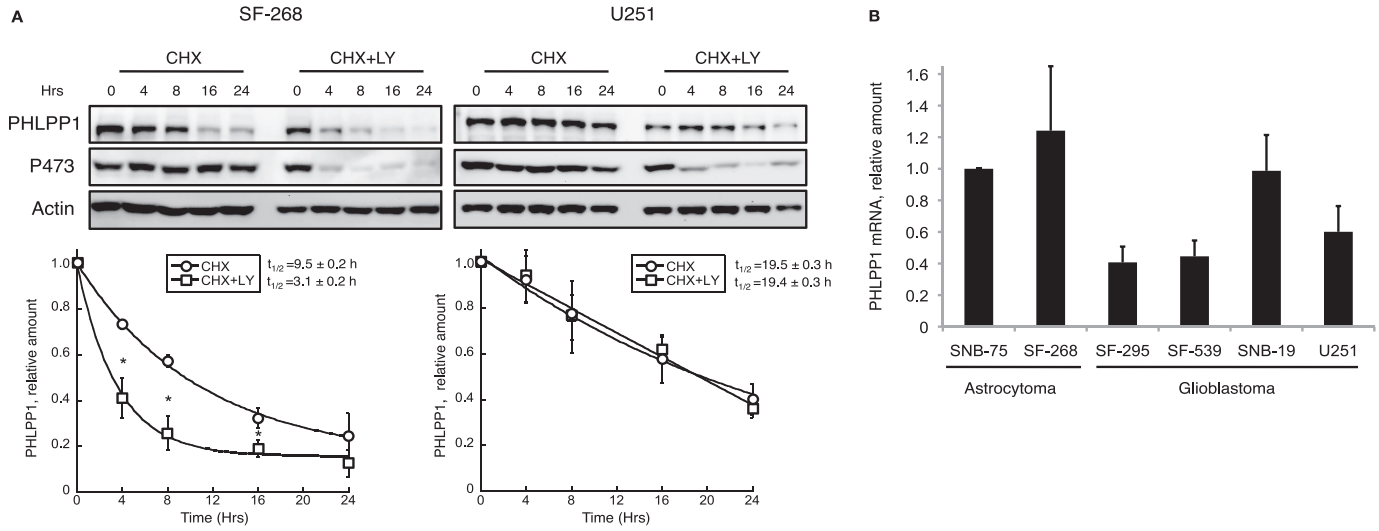


FIGURE 4. **PHLPP1 turnover is slower, and mRNA levels are reduced in glioblastoma cell lines.** *A*, SF-268 (astrocytoma) and U251 (glioblastoma) cell lines were treated with DMSO or LY294002 (20 μM) in addition to cycloheximide (*CHX*; 5 μM). Lysates were collected at the indicated times, and levels of PHLPP1 and the phosphorylation of Akt on Ser-473 (*P473*) were monitored by Western blot analysis. PHLPP1 levels were normalized to actin, which was used as a loading control. Data from three independent experiments were quantified and fit to an exponential decay to obtain half-times of degradation; points represent the average ± S.E. *, $p < 0.05$. *B*, relative PHLPP1 RNA levels were calculated from microarray experiments measured on Affymetrix U95Av2 and U133 arrays, and normalized to the level observed in SNB-75 cells. Data represent the mean ± S.E. of six independent experiments.

tions. Cellular fractionation revealed that PHLPP1 was located primarily in the cytosol of both astrocytoma and glioblastoma cells. Strikingly, β-TrCP1 was primarily cytosolic in the astrocytoma cell lines (Fig. 3*a*, *lanes 3* and *8*) but partitioned almost exclusively in the nuclear fraction of the glioblastoma cells (Fig. 3*a*, *lanes 14*, *19*, and *24*). These data identify a key difference in the cellular localization of β-TrCP1 between cell lines containing an intact *versus* broken feedback loop; PHLPP1 and β-TrCP1 are both located in the cytosol of cells harboring an intact feedback loop, whereas β-TrCP1 is confined to the nucleus, sequestered from PHLPP1, in cells where the feedback loop is broken. Thus, the impaired degradation of PHLPP1 observed in glioblastoma correlates with altered, nuclear localization of β-TrCP1.

To confirm whether our results in cell lines held true in glioblastoma, we obtained tissue samples from humans who died of causes unrelated to CNS tumors (normal brain: frontal cortex, >50% astrocytes) and cell lines derived from neurospheres generated from biopsies of human glioblastoma patients (46, 47). These cells are ideal for studying GBM biology because, unlike cultured cell lines, their genetic profiles are very similar to primary gliomas. Tissue from two healthy donors and five patient-derived glioblastoma tumor neurospheres were homogenized, and cytoplasmic and nuclear fractions were isolated as described under "Experimental Procedures" (Fig. 3*b*). Strikingly, β-TrCP1 was in the nucleus of cells derived from human glioblastoma tumors ($n = 5$; *lanes 6*, *8*, and *10*) but in the cytosolic fraction of normal brain from humans who died of other causes ($n = 2$; *lanes 1* and *2*). To verify the efficacy of our fractionation, annexin 1 and lamin A were used as markers of the cytoplasm and nucleus, respectively. These data confirm that the cellular localization of β-TrCP1 is altered in glioblastoma as compared with healthy human brain.

Turnover of PHLPP1 Is Slower in Glioblastoma Compared with Astrocytoma Cell Lines—The finding that the localization of β-TrCP1 is altered in glioblastoma cell lines led us to ask whether the basal rate of PHLPP1 turnover (*i.e.* independently of Akt inhibition) would be impaired as well in these cells compared with astrocytoma cell lines. SF-268 (intact loop) and U251 (broken loop) cells were treated with cycloheximide (*CHX*), an inhibitor of global protein synthesis, in combination with DMSO or LY294002, and lysates were collected over a 24-h time course (Fig. 4*a*). Quantitative analysis (Fig. 4*a*, *bottom*) of Western blots (Fig. 4*a*, *top*) revealed that under basal conditions PHLPP1 was degraded at a significantly faster rate in the SF-268 cells ($t_{½} = 9.5 \pm 0.2$ h) compared with the U251 cell line ($t_{½} = 19.5 \pm 0.3$ h). As expected, inhibition of PI3K/Akt significantly increased the rate of PHLPP1 turnover in SF-268 cells ($t_{½} = 3.1 \pm 0.2$ h) but had virtually no effect on the rate of turnover in the U251 glioblastoma cell line ($t_{½} = 19.4 \pm 0.3$ h). Furthermore, the rate of basal PHLPP1 turnover in U251 cells was significantly slower than that previously reported, whereas the half-time of PHLPP1 in SF-268 cells was similar to that reported for other cell lines and, additionally, was comparable with that determined for overexpressed PHLPP1 by pulse-chase analysis (data not shown) (44). These data not only indicate that PHLPP1 stability is insensitive to Akt inhibition in glioblastoma but that the basal rate of PHLPP1 turnover is also impaired.

Despite the decreased rate of PHLPP1 turnover in glioblastoma compared with astrocytoma cell lines, the absolute levels of PHLPP1 are similar in both (Fig. 1*a*). Steady-state levels are dictated by the rate of biosynthesis and the rate of degradation. Because the rate of degradation is markedly reduced in the glioblastoma yet steady-state levels are not significantly increased, we reasoned that the rate of biosynthesis may be slower. This led us to examine whether the PHLPP1 mRNA levels differed in glioblastoma *versus* astrocytoma cell lines. To this end, we analyzed data generated from six separate microarray experiments measured on Affymetrix U95Av2 and U133 arrays (supplemental Table 2). Raw data from each experiment were normalized to the level of PHLPP1 mRNA present in the SNB-75 astrocy-

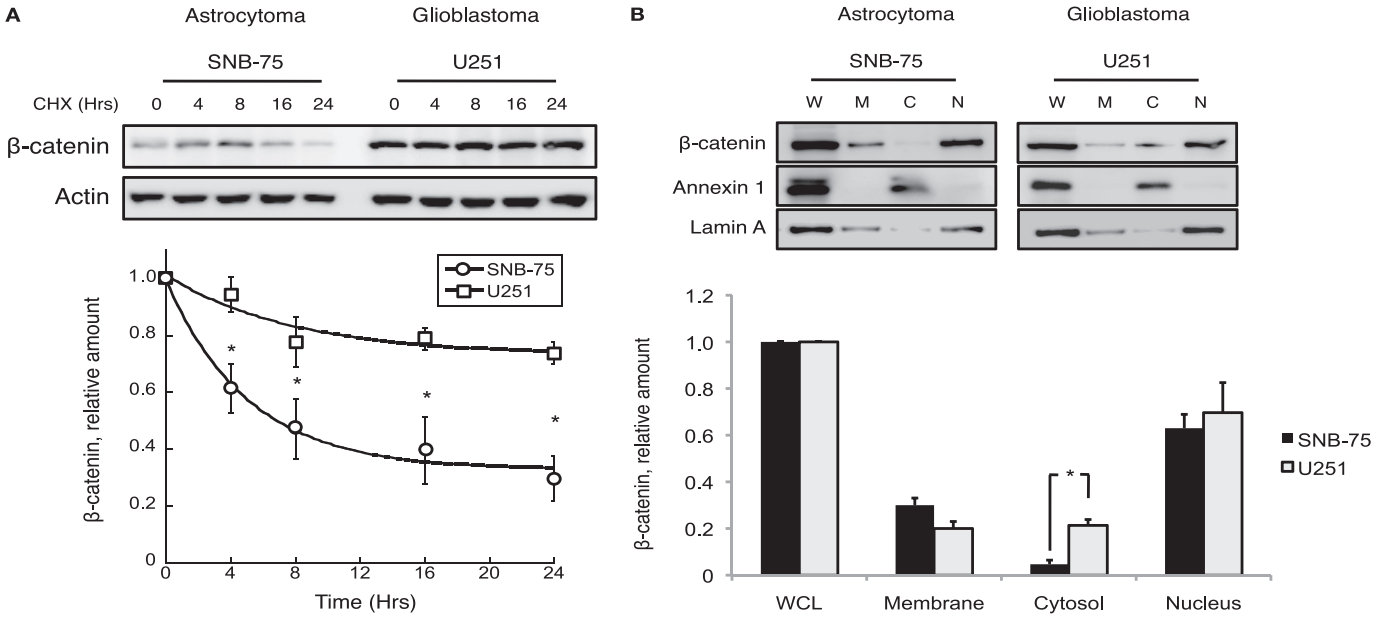


FIGURE 5. **β-Catenin turnover is impaired in glioblastoma cell lines.** *A*, SNB-75 and U251 cell lines were treated with cycloheximide (*CHX*; 5 μM), and lysates were collected over 24 h to monitor the levels of β-catenin and actin. β-Catenin levels were normalized to actin, and data from three independent experiments were quantified and fit to an exponential decay to obtain half-times of degradation; points represent the average ± S.E. *, $p < 0.05$. *B*, SNB-75 and U251 cells were fractionated as described under "Experimental Procedures," and immunoblotting was used to examine β-catenin. Densitometry was used to determine the relative levels of β-catenin in each cellular fraction. The graph represents the average ± S.E. of three independent experiments. *, $p < 0.05$. *W* or *WCL*, whole cell lysate; *M*, membrane; *C*, cytosol; *N*, nuclear.

toma cell line, and relative mRNA levels from each experiment were used to calculate the average level of PHLPP1 mRNA in each cell line. Three of the four glioblastoma cell lines (numbers 56, 57, and 60 in Fig. 1*a*) have at least 40% lower PHLPP1 mRNA levels compared with those in the astrocytoma cell lines (Fig. 4*b*). SNB-19 glioblastoma cells have only slightly less PHLPP1 mRNA compared with SNB-75 cells. Consistent with this cell line having higher PHLPP1 mRNA levels and, thus, a greater rate of PHLPP1 synthesis, the steady-state levels of PHLPP1 are ~40% higher in the SNB-19 cells compared with the other glioblastoma cell lines (number 58, Fig. 1*a*). Taken together, these data indicate that degradation of PHLPP1 is slower in glioblastoma, but steady-state levels of PHLPP1 protein are not significantly increased because of lower mRNA levels.

Turnover of β-Catenin Is Impaired in Glioblastoma Cell Lines—Because the basal rate of PHLPP1 turnover is impaired in glioblastoma cell lines, we asked whether other substrates of β-TrCP1 that are known to be degraded primarily in the cytosol are also stabilized. Previous reports reveal that the level and activity of one such substrate, β-catenin, are frequently enhanced in glioblastoma and correlate with increasing tumor grade and poor patient prognosis (49). Thus, we asked whether β-catenin stability was altered in cell lines where β-TrCP1 is confined to the nucleus. We monitored the rate of degradation of β-catenin in SNB-75 (intact loop) and U251 (broken loop) cell lines by cycloheximide chase over 24 h. Quantitative analysis (Fig. 5*a*, *bottom*) of Western blots (Fig. 5*a*, *top*) revealed that under basal conditions β-catenin was degraded at a significantly faster rate in the SNB-75 cells compared with U251 cells. These data indicate that the degradation of another β-TrCP1 substrate is also impaired in glioblastoma cell lines.

Next, we sought to determine whether the amount of β-catenin in the cytosolic pool is enhanced in glioblastoma cell lines. Indeed, each of the glioblastoma cell lines examined had higher levels of total β-catenin protein compared with astrocytoma cells despite having similar mRNA levels (data not shown and Fig. 2*b*, assayed by densitometry). To measure the relative amount of β-catenin present in each cellular compartment, SNB-75 cells and U251 cells were fractionated as described previously, and equal portions of starting material were analyzed by Western blotting. Cytosolic levels of β-catenin were ~4-fold higher in U251 cells compared with SNB-75 cells (Fig. 5*b*). Thus, the mislocalization of β-TrCP1 in glioblastoma decreases the rate of degradation and leads to accumulation of β-catenin in the cytosol.

Expression of Cytoplasmic β-TrCP1 in Glioblastoma Rescues Akt-mediated Regulation of PHLPP1—The finding that β-TrCP1 is differentially localized in cell lines that have an intact feedback loop compared with those with a broken feedback loop led us to test whether re-introducing β-TrCP1 to the cytoplasm of glioblastoma cells was sufficient to restore the ability of Akt activity to regulate PHLPP1. First, we examined the subcellular location of CFP-tagged β-TrCP1 to determine whether the protein partitions to the cytosol when overexpressed in glioblastoma cells. Fluorescence microscopy and biochemical fractionation revealed exogenously expressed β-TrCP1 in both the cytosol and nucleus of all cell lines. A representative image of U251 glioblastoma cells transfected with CFP-β-TrCP1 illustrates that this protein was localized throughout the cell, with a majority present in the cytosol (Fig. 6*a*). It is noteworthy that the level of exogenous β-TrCP1 was ~3-fold higher than the endogenous protein, which may account for the artificial cytosolic localization observed with overexpression in glioblastoma (data not shown). To test whether cytosolic localization of β-TrCP1 would rescue the ability of Akt activity to regulate PHLPP1 levels, both astrocy-

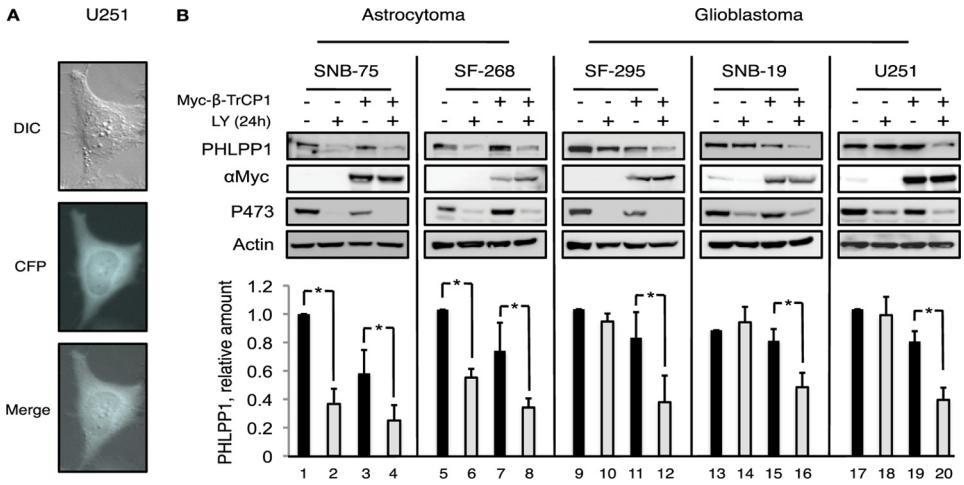


FIGURE 6. **Expression of β-TrCP1 in the cytosol rescues Akt-mediated regulation of PHLPP1 in glioblastoma.** *A*, a CFP-tagged construct of β-TrCP1 was expressed in U251 cells. Differential interference contrast (*DIC*; *top*) and CFP (*middle*) images were merged (*bottom*) to show the cellular localization of exogenous β-TrCP1 in GBM cells. *B*, Myc-β-TrCP1 was overexpressed in astrocytoma and glioblastoma cell lines for 24 h before 24 h treatment with DMSO or LY294002 (20 μM). Immunoblotting was used to monitor the levels of exogenous Myc-β-TrCP1 (*αMyc*), PHLPP1, Akt phosphorylation on Ser-473 (*P473*), and actin. The relative amount of PHLPP1, normalized to actin, is shown in the graph; data represent the mean ± S.E. of three independent experiments. *, $p < 0.05$.

toma and glioblastoma cell lines were transfected with vector or Myc-β-TrCP1 for 24 h before treatment with DMSO or LY294002 for an additional 24 h (Fig. 6*b*). As expected, treatment with LY294002 significantly decreased PHLPP1 levels in the astrocytoma cell lines (*e.g.* compare *lane 1* to *lane 2*). Also, overexpression of β-TrCP1 in cells with an intact feedback loop led to a modest decrease in the level of PHLPP1 (compare *lane 1* and *lane 3*). Moreover, inhibition of PI3K in combination with overexpression of β-TrCP1 reduced PHLPP1 levels below that observed with either β-TrCP1 or LY294002 alone (compare *lanes 2* through *4*). Next, focusing on the glioblastoma cell lines, we verified that treatment with LY294002 alone had no effect on the stability of PHLPP1 (*e.g.* compare *lanes 9* and *10*). However, overexpression of β-TrCP1 alone in cells with a broken feedback loop resulted in a modest decrease in PHLPP1 levels, comparable with that observed in the astrocytoma cell lines (*e.g.* compare *lanes 9* and *11*). Importantly, expression of cytoplasmic β-TrCP1 in combination with PI3K inhibition in the glioblastoma cell lines decreased PHLPP1 protein levels to a similar extent as that observed in astrocytoma cells under the same conditions (*e.g.* compare *lanes 3* and *4* to *11* and *12*). Therefore, restoring the cytoplasmic localization of β-TrCP1 in glioblastoma is sufficient to rescue the defective regulatory feedback loop from Akt to PHLPP1. Taken together these data indicate 1) β-TrCP1 must be present in the cytosol to effectively degrade PHLPP1 and 2) nuclear confinement of β-TrCP1 is responsible for the loss of the feedback loop between Akt and PHLPP1 in glioblastoma.

DISCUSSION

Tightly controlled regulation of protein biosynthesis and degradation in multiple signal transduction pathways plays a central role in maintaining cellular homeostasis. Here we show that β-TrCP1, an E3 ligase crucial to maintaining homeostasis in the PI3K/Akt and Wnt/β-catenin signaling pathways, is confined to the nucleus in glioblastoma and thus spatially segregated from, and unable to properly target, cytosolic substrates for degradation. The altered localization of β-TrCP1 in glioblastoma uncouples the level of a negative regulator, PHLPP, from the level of activated substrate, phospho-Akt. Furthermore, we show that the degradation of β-catenin, a critical mediator of cell survival, is also impaired in glioblastoma cell lines. The finding that β-TrCP1 is localized differently in glioblastoma *versus* normal brain tissue is significant because this E3 ligase controls the levels of several crucial mediators of cell survival and may contribute to the pathogenesis of glioblastoma, a disease for which effective treatment options remain elusive.

The feedback loop that sets the level of PHLPP1 to match the level of phospho-Akt was identified recently by Gao and coworkers (44). Specifically, they showed that GSK-3β, which is inhibited by Akt phosphorylation, directly phosphorylates PHLPP1α to initiate ubiquitination by the $SCF^{\beta\text{-}TrCP1}$ complex and subsequent degradation of ubiquitinated PHLPP1. Thus, the level of substrate, phosphorylated Akt, sets the level of enzyme, PHLPP1; this feed-forward stimulation of its negative regulator, PHLPP1, serves to inhibit its own activity and thus maintain homeostasis. We reasoned that disruption of the Akt-mediated feedback loop on PHLPP1 is a potential mechanism to promote the constitutive activation of Akt that is common in cancer. To test this possibility, we examined levels of PHLPP1 and phospho-Akt in tumor lysates from the NCI60 panel of tumor cell lines to identify candidate lines in which this feedback loop might be broken (Fig. 1*a*). Consistent with Akt activity controlling PHLPP1 levels, we observed a modest correlation between the relative levels of basal Akt activity (assessed by monitoring the phosphorylation of Thr-308) and PHLPP1 protein levels. Interestingly, this correlation was lost in four of six CNS tumor cell lines. Furthermore, the loss was associated with tumor grade; the four lines in which PHLPP1:Akt (Thr-308) ratio was unusually low were from grade IV glioblastomas, whereas the remaining two CNS tumor lines, with an average or above average PHLPP1:Akt (Thr-308) ratio, were derived from lower grade astrocytomas (Fig. 1*b*). One possible explanation for the dramatic divergence between Akt activity and PHLPP1

levels is that the feedback loop was lost in the cancer cell lines derived from more aggressive glioblastomas. We tested this by examining whether PHLPP1 levels were sensitive to inhibition of PI3K in all of the CNS tumor cell lines. Indeed, PHLPP1 levels were unchanged after inhibition of PI3K/Akt in all four lines derived from glioblastoma but were sensitive to inhibition of PI3K in the astrocytoma cell lines in which phospho-Akt and PHLPP1 levels predicted an intact loop (Fig. 1*c*). These data are consistent with a model in which Akt-mediated regulation of PHLPP1 stability is lost in aggressive glioblastomas but not in less aggressive astrocytomas. Thus, we have identified a subset of high grade tumors that have lost the negative feedback loop between PHLPP1 and Akt, implying that this event may be an important factor in the progression of this disease.

Interestingly, we found that the turnover of PHLPP1 is noticeably slower in U251 cells (broken loop) compared with that in SF-268 (intact loop) in which the half-time of PHLPP1 turnover is similar to that previously reported (Fig. 4*a*) (44). This suggests that there is a defect in basal PHLPP1 degradation that is independent of agonist-evoked signaling. We investigated each step in the process of PHLPP1 degradation and determined that neither mutation of PHLPP1 nor inactivation of the kinases responsible for preparing it for ubiquitination is observed in glioblastoma (Fig. 2*b*). Instead, cellular fractionation revealed that β-TrCP1, the E3-ligase responsible for targeting PHLPP1 for degradation, is differentially localized in astrocytomas *versus* glioblastomas. In astrocytoma cell lines containing an intact feedback loop, both β-TrCP1 and PHLPP1 are primarily located in the cytosol. However, although PHLPP1 is in the cytosol of glioblastoma cells, β-TrCP1 is confined to the nucleus. These data suggest that under normal circumstances (intact feedback loop), when PHLPP1 is phosphorylated by CK1 and GSK-3β, it is then bound and ubiquitinated by the $SCF^{\beta\text{-}TrCP1}$ complex, leading to degradation by the proteasome. However, in glioblastoma, although PHLPP1 is properly phosphorylated by upstream kinases, it is spatially segregated from β-TrCP1, and thus PHLPP1 can no longer interact with β-TrCP1 and is not as readily degraded. Consistent with this, reintroduction of β-TrCP1 to the cytosol of glioblastoma cells is sufficient to restore the ability of Akt to control PHLPP1 levels. Therefore, PHLPP1 levels are not properly regulated in glioblastoma under basal or agonist-stimulated conditions because of the altered, nuclear localization of β-TrCP1.

Curiously, our findings reveal that PHLPP1, although uncoupled from Akt, is actually more stable in glioblastoma compared with astrocytoma. This suggests that loss of the feedback loop does not modulate PHLPP levels in a manner that could account for the constitutive activation of Akt, as was our initial hypothesis. Thus, our work establishes that PHLPP1 levels are insensitive to PI3K inhibitors in glioblastoma, but a compensating mechanism stabilizes the protein. Interestingly, analysis of PHLPP1 mRNA levels showed that in a majority of the glioblastoma cell lines tested there is significantly less PHLPP1 mRNA compared with the astrocytoma cell lines. These data explain why, despite the decreased rate of degradation in glioblastoma, steady-state levels of PHLPP1 protein are similar to those detected in the astrocytoma cell lines (Fig. 4) and ultimately why these cell lines were identified from our screen of the NCI60. To date little is known about how PHLPP is regulated at the transcriptional level. β-TrCP1 is known to affect the transcription of various proteins through modulation of its substrates, most notably the T-cell specific transcription factor/lymphoid enhancer binding factor (TCF/LEF), ATF/cAMP-response element-binding protein (CREB), and NF-κB transcription factors (50–52). Therefore, it is an intriguing possibility that β-TrCP1 could influence both the biosynthesis (RNA levels) and degradation of PHLPP1. Importantly, our data reveal that inhibition of the PI3K pathway in glioblastoma, in contrast to astrocytoma, will not reduce PHLPP1 levels.

Previous reports from both immunohistochemical and overexpression studies have revealed primarily cytoplasmic localization of β-TrCP1 (53–55), consistent with the cytosolic localization of the majority of its substrates. Here, we find that in healthy human brain, β-TrCP1 is localized to the cytosol, whereas nuclear accumulation is associated with high grade glioblastoma (Fig. 3). To our knowledge this is the first report to show that endogenous β-TrCP1 is cytosolic in the human brain and mislocalized to the nucleus in human glioblastoma tumors. To date we have been unable to establish whether endogenous Homolog of Slimb(HOS)/β-TrCP2, a functionally redundant β-TrCP isoform, is also confined to the nucleus in glioblastoma due to a lack of suitable reagents. However, given the degradation of multiple shared substrates of β-TrCPs is impaired, it is clear that if β-TrCP2 is cytosolic, it is unable to compensate for the loss of β-TrCP1 in the cytosol. The mechanism responsible for the nuclear confinement of β-TrCP1 in glioblastoma remains unknown, as there are numerous possible culprits. β-TrCP1 does not contain a nuclear localization signal and, therefore, must be localized to the nucleus through interactions with other proteins. It is possible that increased affinity or abundance of a nuclear protein could sequester β-TrCP1 in the nuclear compartment. In fact, it has been reported that β-TrCP1 is primarily nuclear in some cell types because of constitutive binding to the nuclear phosphoprotein, heterogeneous nuclear ribonucleoprotein U (hnRNP-U) (56). Recent work has also shown that differential splicing of β-TrCP1 can result in dramatic changes in cellular localization and biological roles (57). Our model is consistent with nuclear localization of β-TrCP1 impairing its ability to target its cytosolic substrates for degradation. Thus, a more thorough exploration of the mechanisms governing β-TrCP localization may be significant in understanding the activation of signal transduction pathways influenced by its substrates.

The finding that β-TrCP1 is confined to the nucleus and cannot properly regulate the cytosolic degradation of substrates is particularly intriguing considering that several of the proteins β-TrCP1 is known to regulate are linked to tumor progression (40). Here we demonstrate that the cytosolic pool of β-catenin is degraded at a slower rate in glioblastoma cell lines where β-TrCP1 is confined to the nucleus (Fig. 4). There is precedent for the induction of β-catenin in the progression of tumors, specifically those of the CNS. Immunohistochemical studies comparing β-catenin levels in glioma patient samples to those in normal brain tissue reveal that β-catenin levels are higher in glioma samples, correlating with poor patient prognosis and outcome, yet relative mRNA levels remain un-

changed, supporting a defect in protein degradation (49, 58). Another study of glioblastoma patient samples reported intense, homogenous staining of β-catenin in the cytoplasm of 81% (26/32) of samples (59). Additionally, a study of 45 astrocytic gliomas showed a correlation between increased expression of β-catenin and ascending order of the tumor grade (60). Over 80% of glioblastoma (9/11) displayed high immunoreactivity for β-catenin, yet only two of these stained positively for nuclear accumulation, indicating that a majority of the cytoplasmic β-catenin is not being properly degraded. It is also of note that mutations of β-catenin that would render it refractory to degradation are very rare in brain tumors, suggesting another aberration is responsible for accumulation of β-catenin in tumorigenesis (61, 62). Taken together with our findings, altered localization of β-TrCP1 in glioblastoma is potentially responsible for the unusually high levels of β-catenin observed in high grade glioma patient samples. Therefore, in addition to loss of PHLPP1 regulation, the stabilization of oncogenic factors arising from nuclear localization of β-TrCP1 may be favorably selected for in late stage tumors.

In summary, we have identified a subset of CNS tumors that display a distinct cellular localization of β-TrCP1. Our finding that the cellular localization of β-TrCP1 is altered in glioblastoma contributes to the dysregulation of two signal transduction pathways that are critical in tumorigenesis. Mislocalization of β-TrCP1 in glioblastoma not only impairs the ability of Akt to regulate PHLPP1 stability (rendering PHLPP levels insensitive to inhibition of the PI3K pathway) but also provides a novel mechanism for the amplification of β-catenin often observed in this disease.

Acknowledgments—We thank the Developmental Therapeutics Program, NCI, National Institutes of Health, for providing the NCI60 panel of tumor cell lines, Dr. Eliezer Masliah (University of California at San Diego (UCSD)) and the Alzheimer's Disease Research Center at UCSD (NIH-AG5131) for the human brain tissue, Dr. Cameron Brennan (Memorial Sloan-Kettering Cancer Center) for providing TS600 and TS576 neurosphere cell lines, and Kenny McLeod (University of Edinburgh) and Maria Del-Mar Inda (UCSD) for preparation of glioblastoma neurosphere cell lines.

REFERENCES

1. Ohgaki, H., and Kleihues, P. (2007) *Am. J. Pathol.* **170,** 1445–1453
2. Furnari, F. B., Fenton, T., Bachoo, R. M., Mukasa, A., Stommel, J. M., Stegh, A., Hahn, W. C., Ligon, K. L., Louis, D. N., Brennan, C., Chin, L., DePinho, R. A., and Cavenee, W. K. (2007) *Genes. Dev.* **21,** 2683–2710
3. Blume-Jensen, P., and Hunter, T. (2001) *Nature* **411,** 355–365
4. Calleja, V., Alcor, D., Laguerre, M., Park, J., Vojnovic, B., Hemmings, B. A., Downward, J., Parker, P. J., and Larijani, B. (2007) *PLoS Biol.* **5,** e95
5. Sarbassov, D. D., Guertin, D. A., Ali, S. M., and Sabatini, D. M. (2005) *Science* **307,** 1098–1101
6. Guertin, D. A., Stevens, D. M., Thoreen, C. C., Burds, A. A., Kalaany, N. Y., Moffat, J., Brown, M., Fitzgerald, K. J., and Sabatini, D. M. (2006) *Dev. Cell* **11,** 859–871
7. Cantley, L. C. (2002) *Science* **296,** 1655–1657
8. Maehama, T., and Dixon, J. E. (1998) *J. Biol. Chem.* **273,** 13375–13378
9. Gao, T., Furnari, F., and Newton, A. C. (2005) *Mol. Cell* **18,** 13–24
10. Brognard, J., Sierecki, E., Gao, T., and Newton, A. C. (2007) *Mol. Cell* **25,** 917–931
11. Giles, R. H., van Es, J. H., and Clevers, H. (2003) *Biochim. Biophys. Acta* **1653,** 1–24
12. Hasselblatt, P., Gresh, L., Kudo, H., Guinea-Viniegra, J., and Wagner, E. F. (2008) *Oncogene* **27,** 6102–6109
13. Moon, R. T., Kohn, A. D., De Ferrari, G. V., and Kaykas, A. (2004) *Nat. Rev. Genet.* **5,** 691–701
14. Vogt, P. K., Kang, S., Elsliger, M. A., and Gymnopoulos, M. (2007) *Trends Biochem. Sci.* **32,** 342–349
15. Li, J., Yen, C., Liaw, D., Podsypanina, K., Bose, S., Wang, S. I., Puc, J., Miliaresis, C., Rodgers, L., McCombie, R., Bigner, S. H., Giovanella, B. C., Ittmann, M., Tycko, B., Hibshoosh, H., Wigler, M. H., and Parsons, R. (1997) *Science* **275,** 1943–1947
16. Samuels, Y., Diaz, L. A., Jr., Schmidt-Kittler, O., Cummins, J. M., Delong, L., Cheong, I., Rago, C., Huso, D. L., Lengauer, C., Kinzler, K. W., Vogelstein, B., and Velculescu, V. E. (2005) *Cancer Cell* **7,** 561–573
17. Slamon, D. J., Clark, G. M., Wong, S. G., Levin, W. J., Ullrich, A., and McGuire, W. L. (1987) *Science* **235,** 177–182
18. Park, W. S., Oh, R. R., Park, J. Y., Lee, S. H., Shin, M. S., Kim, Y. S., Kim, S. Y., Lee, H. K., Kim, P. J., Oh, S. T., Yoo, N. J., and Lee, J. Y. (1999) *Cancer Res.* **59,** 4257–4260
19. Powell, S. M., Zilz, N., Beazer-Barclay, Y., Bryan, T. M., Hamilton, S. R., Thibodeau, S. N., Vogelstein, B., and Kinzler, K. W. (1992) *Nature* **359,** 235–237
20. Uematsu, K., He, B., You, L., Xu, Z., McCormick, F., and Jablons, D. M. (2003) *Oncogene* **22,** 7218–7221
21. Brognard, J., and Newton, A. C. (2008) *Trends Endocrinol. Metab.* **19,** 223–230
22. Gao, T., Brognard, J., and Newton, A. C. (2008) *J. Biol. Chem.* **283,** 6300–6311
23. Basso, K., Margolin, A. A., Stolovitzky, G., Klein, U., Dalla-Favera, R., and Califano, A. (2005) *Nat. Genet.* **37,** 382–390
24. Ouillette, P., Erba, H., Kujawski, L., Kaminski, M., Shedden, K., and Malek, S. N. (2008) *Cancer Res.* **68,** 1012–1021
25. Hao, Y., Triadafilopoulos, G., Sahbaie, P., Young, H. S., Omary, M. B., and Lowe, A. W. (2006) *Gastroenterology* **131,** 925–933
26. Sabates-Bellver, J., Van der Flier, L. G., de Palo, M., Cattaneo, E., Maake, C., Rehrauer, H., Laczko, E., Kurowski, M. A., Bujnicki, J. M., Menigatti, M., Luz, J., Ranalli, T. V., Gomes, V., Pastorelli, A., Faggiani, R., Anti, M., Jiricny, J., Clevers, H., and Marra, G. (2007) *Mol. Cancer Res.* **5,** 1263–1275
27. Kaiser, S., Park, Y. K., Franklin, J. L., Halberg, R. B., Yu, M., Jessen, W. J., Freudenberg, J., Chen, X., Haigis, K., Jegga, A. G., Kong, S., Sakthivel, B., Xu, H., Reichling, T., Azhar, M., Boivin, G. P., Roberts, R. B., Bissahoyo, A. C., Gonzales, F., Bloom, G. C., Eschrich, S., Carter, S. L., Aronow, J. E., Kleimeyer, J., Kleimeyer, M., Ramaswamy, V., Settle, S. H., Boone, B., Levy, S., Graff, J. M., Doetschman, T., Groden, J., Dove, W. F., Threadgill, D. W., Yeatman, T. J., Coffey, R. J., Jr., and Aronow, B. J. (2007) *Genome Biol.* **8,** R131
28. Gaspar, C., Cardoso, J., Franken, P., Molenaar, L., Morreau, H., Möslein, G., Sampson, J., Boer, J. M., de Menezes, R. X., and Fodde, R. (2008) *Am. J. Pathol.* **172,** 1363–1380
29. Bredel, M., Bredel, C., Juric, D., Harsh, G. R., Vogel, H., Recht, L. D., and Sikic, B. I. (2005) *Cancer Res.* **65,** 4088–4096
30. Talantov, D., Mazumder, A., Yu, J. X., Briggs, T., Jiang, Y., Backus, J., Atkins, D., and Wang, Y. (2005) *Clin. Cancer Res.* **11,** 7234–7242
31. Karnoub, A. E., Dash, A. B., Vo, A. P., Sullivan, A., Brooks, M. W., Bell, G. W., Richardson, A. L., Polyak, K., Tubo, R., and Weinberg, R. A. (2007) *Nature* **449,** 557–563
32. Richardson, A. L., Wang, Z. C., De Nicolo, A., Lu, X., Brown, M., Miron, A., Liao, X., Iglehart, J. D., Livingston, D. M., and Ganesan, S. (2006) *Cancer Cell* **9,** 121–132
33. Qiao, M., Wang, Y., Xu, X., Lu, J., Dong, Y., Tao, W., Stein, J., Stein, G. S., Iglehart, J. D., Shi, Q., and Pardee, A. B. (2010) *Mol. Cell* **38,** 512–523
34. Liu, J., Weiss, H. L., Rychahou, P., Jackson, L. N., Evers, B. M., and Gao, T. (2009) *Oncogene* **28,** 994–1004
35. Taylor, B. S., Schultz, N., Hieronymus, H., Gopalan, A., Xiao, Y., Carver, B. S., Arora, V. K., Kaushik, P., Cerami, E., Reva, B., Antipin, Y., Mitsiades, N., Landers, T., Dolgalev, I., Major, J. E., Wilson, M., Socci, N. D., Lash, A. E., Heguy, A., Eastham, J. A., Scher, H. I., Reuter, V. E., Scardino, P. T., Sander, C., Sawyers, C. L., and Gerald, W. L. (2010) *Cancer Cell* **18,** 11–22

36. Network, T. C. G. A. R. (2008) *Nature* **455,** 1061–1068
37. Parsons, D. W., Jones, S., Zhang, X., Lin, J. C., Leary, R. J., Angenendt, P., Mankoo, P., Carter, H., Siu, I. M., Gallia, G. L., Olivi, A., McLendon, R., Rasheed, B. A., Keir, S., Nikolskaya, T., Nikolsky, Y., Busam, D. A., Tekleab, H., Diaz, L. A., Jr., Hartigan, J., Smith, D. R., Strausberg, R. L., Marie, S. K., Shinjo, S. M., Yan, H., Riggins, G. J., Bigner, D. D., Karchin, R., Papadopoulos, N., Parmigiani, G., Vogelstein, B., Velculescu, V. E., and Kinzler, K. W. (2008) *Science* **321,** 1807–1812
38. Joazeiro, C. A., and Hunter, T. (2000) *Science* **289,** 2061–2062
39. Frescas, D., and Pagano, M. (2008) *Nat. Rev. Cancer* **8,** 438–449
40. Fuchs, S. Y., Spiegelman, V. S., and Kumar, K. G. (2004) *Oncogene* **23,** 2028–2036
41. Aberle, H., Bauer, A., Stappert, J., Kispert, A., and Kemler, R. (1997) *EMBO J.* **16,** 3797–3804
42. Hart, M., Concordet, J. P., Lassot, I., Albert, I., del los Santos, R., Durand, H., Perret, C., Rubinfeld, B., Margottin, F., Benarous, R., and Polakis, P. (1999) *Curr. Biol.* **9,** 207–210
43. Yaron, A., Hatzubai, A., Davis, M., Lavon, I., Amit, S., Manning, A. M., Andersen, J. S., Mann, M., Mercurio, F., and Ben-Neriah, Y. (1998) *Nature* **396,** 590–594
44. Li, X., Liu, J., and Gao, T. (2009) *Mol. Cell. Biol.* **29,** 6192–6205
45. Ohta, T., and Xiong, Y. (2001) *Cancer Res.* **61,** 1347–1353
46. Fael Al-Mayhani, T. M., Ball, S. L., Zhao, J. W., Fawcett, J., Ichimura, K., Collins, P. V., and Watts, C. (2009) *J. Neurosci. Methods* **176,** 192–199
47. Pollard, S. M., Yoshikawa, K., Clarke, I. D., Danovi, D., Stricker, S., Russell, R., Bayani, J., Head, R., Lee, M., Bernstein, M., Squire, J. A., Smith, A., and Dirks, P. (2009) *Cell Stem Cell* **4,** 568–580
48. Inda, M. M., Bonavia, R., Mukasa, A., Narita, Y., Sah, D. W., Vandenberg, S., Brennan, C., Johns, T. G., Bachoo, R., Hadwiger, P., Tan, P., Depinho, R. A., Cavenee, W., and Furnari, F. (2010) *Genes. Dev.* **24,** 1731–1745
49. Liu, C., Tu, Y., Sun, X., Jiang, J., Jin, X., Bo, X., Li, Z., Bian, A., Wang, X., Liu, D., Wang, Z., and Ding, L. (2010) *Clin. Exp. Med.*, in press
50. Lassot, I., Ségéral, E., Berlioz-Torrent, C., Durand, H., Groussin, L., Hai, T., Benarous, R., and Margottin-Goguet, F. (2001) *Mol. Cell. Biol.* **21,** 2192–2202
51. Peifer, M., and Polakis, P. (2000) *Science* **287,** 1606–1609
52. Karin, M. (1999) *J. Biol. Chem.* **274,** 27339–27342
53. Winston, J. T., Strack, P., Beer-Romero, P., Chu, C. Y., Elledge, S. J., and Harper, J. W. (1999) *Genes. Dev.* **13,** 270–283
54. Mantovani, F., and Banks, L. (2003) *J. Biol. Chem.* **278,** 42477–42486
55. Ougolkov, A., Zhang, B., Yamashita, K., Bilim, V., Mai, M., Fuchs, S. Y., and Minamoto, T. (2004) *J. Natl. Cancer Inst.* **96,** 1161–1170
56. Davis, M., Hatzubai, A., Andersen, J. S., Ben-Shushan, E., Fisher, G. Z., Yaron, A., Bauskin, A., Mercurio, F., Mann, M., and Ben-Neriah, Y. (2002) *Genes. Dev.* **16,** 439–451
57. Seo, E., Kim, H., Kim, R., Yun, S., Kim, M., Han, J. K., Costantini, F., and Jho, E. H. (2009) *Cell. Signal.* **21,** 43–51
58. Pu, P., Zhang, Z., Kang, C., Jiang, R., Jia, Z., Wang, G., and Jiang, H. (2009) *Cancer Gene. Ther.* **16,** 351–361
59. Yano, H., Hara, A., Takenaka, K., Nakatani, K., Shinoda, J., Shimokawa, K., Yoshimi, N., Mori, H., and Sakai, N. (2000) *Neurol. Res.* **22,** 650–656
60. Utsuki, S., Sato, Y., Oka, H., Tsuchiya, B., Suzuki, S., and Fujii, K. (2002) *J. Neurooncol.* **57,** 187–192
61. Yokota, N., Nishizawa, S., Ohta, S., Date, H., Sugimura, H., Namba, H., and Maekawa, M. (2002) *Int. J. Cancer* **101,** 198–201
62. Gilbertson, R. J. (2004) *Lancet Oncol.* **5,** 209–218